Effect of the Electroacupuncture at ST36 in TMT-induced Memory Deficit Rats

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In order to the neuroprotective effect of electroacupuncture (EA), the present study examined the effects of electroacupuncture inacupoint ST36 (Stomach 36) on trimethyltin chloride (TMT)-induced cognitive impairments rat using the Morris water maze (MWM) task and immunohistochemistry staining. The rats were randomly divided into the following groups: naïve rat (Normal), TMT injection rat (Control), TMT injection + EA treated rat inacupoint ST36 (ST36) and TMT injection + EA treated rat in non-acupoint, base of tail (Non-AC). Electroacupuncture (2Hz, 2mA, and 10 minutes)was applied either to the acupuncture point ST36 or the nonacupuncture point in the tail for the last 14 days. In the water maze test, the animals were trained to find a platform in a fixed position during 4d and then received 60s probe trial on the 5th day following removal of platform from the pool. Rats with TMT injection showed impaired learning and memory of the tasks and treatment with EA in acupoint ST36 ($P<0.05$) produced a significant improvement in escape latency to find the platform after 2nd day and retention trial in the Morris water maze. Consistent with behavioral data, treatment with EA in acupoint ST36 also significantly increased expression of Choline acetyltransferase (ChAT) and Acetylcholinesterase (AChE) immunoreactive neurons in the hippocampus compared to the Control group. These results demonstrated that EA in acupoint ST36 has a protective effect against TMT-induced neuronal and cognitive impairments. The present study suggests that EA in acupoint ST36 might be useful in the treatment of TMT-induced learning and memory deficit.

Key words : ST36 (Stomach 36), Trimethyltin chloride (TMT), Morris water maze, Cholineacetyl transferase (ChAT), Acetylcholine esterase (AChE)

Introduction

The organotin compound trimethyltin chloride (C3H9ClSn) (TMT) is a potent neurotoxicant that selectively induces neuronal death in both human and animal limbic system, and inparticular in the hippocampal formation. This substance is regarded as being particularly useful for studying the response to injury on account of the distinct pattern of degeneration it causes in rodent brain. In rats, TMT induces the degeneration of pyramidal neurons in the hippocampus and cortical areas (pyriform cortex, entorhinal cortex, subiculum) connected to the hippocampus, but there is also neuronal loss in the association areas[1-7]. Also, in experimental animals TMT administration induces seizures, behavioural alterations (hyperactivity, tail mutilation, vocalisation and hyperexcitability and aggression) and cognitive deficits (memory loss and learning impairment) referred to severe hippocampal damage[1,3,8]. TMT intoxication impairs the acquisition of water maze and biel maze (water avoidance) task as well as Hebb-Williams maze and radial arm maze performance and passive avoidance test[9-14].

Many studies have shown that acupuncture can help regulate the deficit in learning and memory. For example, it has been shown that acupuncture needles at certain acupoints can improve the learning and memory ability of the model rats with AD. The mechanism of action might be related to the decreasing cholinergic neuron damage[15]. Acupuncture has been also used for the enhancement of functional recovery from various disorders. It has been shown to be effective for analgesia, promotion of homeostasis, and changes in the microcirculatory network as well as improvements in brain circulation[16-19]. Acupuncture treatment has been particularly effective for streptozotocin-induced Alzheimer's disease and

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· Received : 2011/07/13 · Revised : 2011/08/04 · Accepted : 2011/08/10

hippocampal LTP[20,21]. Although many studies have indicated that acupuncture provides a neuroprotective effect against TMT-induced brain damage, the protective mechanisms are not fully understood.

ST36 (Stomach 36) is one of the most frequently used acupuncture points which appears to be a versatile acupuncture point[22]. The stimulation to ST36 is associated with strengthening the spleen and the stomach, regulating the intestine, and stabilizing the mind. In recent, several studies have reported the memory improvement effect of ST36 in animal models[23,24].

The present study was undertaken to evaluate the neuroprotective effect of electroacupuncture in acupoint ST36 on TMT-induced learning and memory deficits in the rats and to elucidate the mechanism underlying these protective effects in rats. Rats were tested on Morris water maze for the spatial learning and memory. The analyzed parameters included cholineacetyl transferase (ChAT) and acetylcholine esterase (AchE) in the hippocampus and medial septum.

Materials & Methods

1. Animals

All the experimental procedures performed on the animals were conducted with the approval of the Ethics Committee of the Kyung Hee University and in accordance with the US National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH Publication no. 80-23, revised 1996).

Sprague-Dawley rats (Orient Animal Corp, Kyunggido, Korea) that weighed 220-240 g each were used for the experiments. The male rats were group-housed (three per cage) under a reversed light-dark cycle (light on from 08:00-20:00 hr). The room temperature was 20~25℃ and the humidity was 30±5%. The rats had free access to food and water. All the rats were handled daily for at least a week prior to the experiment.

2. Trimetyltin (TMT) injection and Electroacupucture (EA) stimulation

The rats were injected intraperitoneally (i.p.) with TMT (8.0 mg/kg, body weight) dissolved in 0.9% saline and then returned to their home cages. The rats were randomly divided into the following groups: the naïve rat (Normal, n=9), TMT injection rat (Control, n=8), TMT injection + EA treated rat in ST36 (ST36, n=14) and TMT injection + EA treated rat in base of tail (Non-AC, n=14).

Electroacupuncture was applied either to the acupuncture point ST36 or the nonacupuncture point in the tail for last 2 weeks. From the 17th after the treatment of EA, the water maze test was performed for 5 days. The acupuncture needle (26 gauge, stainless steel) was inserted around 3 mm deep into left and right ST36. The ST36 is located approximately 10 mm below the knee joint and about 5 mm lateral from the midline on the anterior surface of the hind leg[25]. The acupuncture needles were connected to the output terminals of a stimulator that delivered square waves of 2 Hz and 2 mA pulse width for 10 minute per day for 14 days[25]. The same parameter of electroacupuncture was applied to rats in the Non-AC group.

3. Morris water maze test

All the animals started training on the MWM task in a swimming pool (1.8 m diameter and 0.5 m high, filled with milky water at a temperature in the 22±2℃ ange) for 5 days. A 12 cm diameter round platform was hidden in a constant location (the quadrant center) within the pool with its top surface submerged 1.5 cm below the water level. The rats were trained to locate the hidden island during three trials per day for 4 days. After the 4 days, they were started in the quadrant opposite to the target and were forced to swim for 60 sec in the pool without a platform. The spatial memory of the rats was assessed as the latency time. The time spent in the training quadrant, i.e., the previous location of the platform, was recorded and used as a measure of memory retention. A video camera was mounted on the ceiling above the pool and it was connected to a video-recorder and tracking device (S-MART; Pan-Lab, Barcelona, Spain), which permitted on-line and off-line automated tracking of the path taken by the animal.

4. ChAT and AChE immunohistochemistry

At the end of the behavioral observation, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and then perfused transcardially with 100 ml of saline, followed by 500 ml of a 4% solution of formaldehyde prepared in phosphate buffer. The brains were then removed, postfixed in the same fixative for two to three hours at 4℃ and then placed overnight at 4℃ in PBS containing 20% sucrose. On the following day, the brain was cut into coronal sections that were sliced to 30μm-thicknesses. The primary rabbit polyclonal antibodies against the following specific antigen were used: cholinacetyl transferase (ChAT, concentration 1:2000; Cambridge Research Biochemicals, Wilmington, DE, U.S.A), acetylcholine esterase (AChE, concentration 1:100; Santacruz biotechnology, Delaware Avenue Santa Cruz, CA, U.S.A). The primary antibody was prepared at a dilution of 2000X in 0.3% PBST, 2% normal rabbit serum and

0.001% kehole limpit hemocyanin (Sigma, USA). The sections were incubated in the primary antiserum for 72 h at 4℃. After three more rinses in PBST, the sections were placed in Vectastain Elite ABC reagent (Vector laboratories, Burlingame, CA) for 2 h at room temperature. Following a further rinsing in PBS, the tissue was developed using diaminobenzadine (sigma, USA) as the chromogen. Images were captured using an Axio Vision 3.0 imaging systems (Zeiss, Oberkochen, Germany) and processed in Adobe Photoshop. For measuring cells of ChAT, the grid was placed on CA1 and CA3 in the hippocampus areas according to the method of Paxinos et al[26]. The number of cells was counted at 100x magnification using a microscope rectangle grid measuring 200x200 μm.

5. Statistical methods

The data is reported as the means S.E.M. of the individual values of the rats from each group. SPSS 18.0 for Windows was used for statistical analysis. The significance level was set at P<0.05. Multiple ANOVAs were used for the statistical analysis of the data from the acquisition test in the Morris water maze at various time points during the test period. One-way ANOVA was used for the retention test in the Morris water maze and AChE-immunohistochemistry, and this way followed by the LSD test.

Results

1. Effect of EA in ST36 on water maze task

Fig. 1(A) shows mean group latencies to reach to hidden platform in the MWM for all groups for 4 days. The escape latency differed among the groups when the results were averaged over all the session. The Control group showed a worse performance than did the Normal group.

To examine the spatial memory of rats, the time spent swimming to the platform was compared and analysis is illustrated in Fig. 1(B). The times spent to the quadrant were significantly different among the groups [F3, 36=7.628, p<0.001] the Normal group spent more time around the platform than the Control group (p<0.01 for the Normal group). However, treatment of EA in ST36 was increased the time spent in the quadrant compared Control group (p<0.05), but not the Non-AC group.

2. ChAT immunoreactive neurons of the hippocampus and medial septum

The results of determining the ChAT immunoreative cells per section from different hippocampal formations and medial septum are shown in Fig. 2(A) and (B). The number of ChAT neurons in the CA1 area was 27.0±3.5 in the Normal group, 9.5±2.3 in the Control group, 16.8±1.8 in the ST36 group and 11.2±1.6 in the Non-AC group [F3,17=10.287, p<0.001]. The ChAT immunoreactive cells in the CA3 area were 27.5±3.7 in the Normal group, 11.0±0.7 in the Control group, 17.6±1.7in the ST36 group and 9.5±1.8 in the Non-AC group [F3,19=13.629, p<0.001]. Also, the ChAT immunoreactive cells in the medial septum were 16.8±1.8 in the Normal group, 6.2±0.3 in the Control group, 17.5±2.68 in the ST36 group and 7.3±1.6 in the Non-AC group [F3,17=11.117, p<0.001]. The number of ChAT positive neurons was significantly increased in the ST36 group compared to the Control group (p<0.001), but not the Non-AC group.

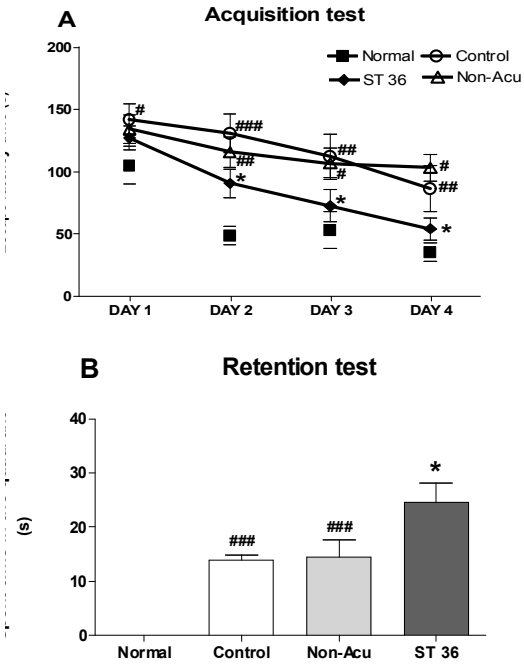

Fig. 1. (A) The latency to escape onto the hidden platform during the Morris water maze. The task was performed with 3 trials per day during 4 days for the acquisition test. The values are presented as means±S.E.M. #p<0.05, ##p<0.01 ###p<0.001 vs. normal group & *p<0.05 vs. control group, respectively. **(B) Retention performance was tested on 5th day.** The rats received a 1 min probe trial in which the platform was removed from the pool for retention testing. The values are presented as means±S.E.M. ###p<0.05 vs. normal group & *p<0.05 vs. control group, respectively.

3. AChE immunoreactive neurons of the hippocampus

The results of determining the AChE immunoreative cells per section from different hippocampal formations are shown in Fig. 3(A) and (B). The density of AChE-ir neurons (% of relative Normal group) in the CA1 area was 78.8±3.2 in the Control group, 94.7±7.6 in the ST36 group and 76.5±2.8 in the Non-AC group [F3,15=7.193, p<0.01]. Also, the AChE-ir neurons in the CA3 area were 82.9±4.2 in the Control group, 86.8±3.77 in the ST36 group and 93.1±17.3 in the Non-AC

group [F3,16=2.704]. The density of AChE positive neurons in the CA1 area was significantly increased in the ST36 group compared to the Control group ($p<0.01$), but not the Non-AC group.

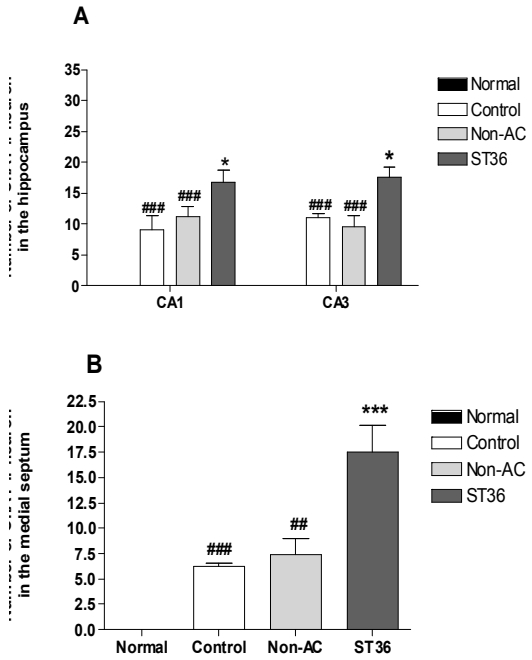

Fig. 2. (A) The number of choline acetyltransferase (ChAT) immunostained nuclei in different hippocampual CA1 and CA3 medial septumof the experimental groups. (B) The number of choline acetyltransferase (ChAT) immunostained nuclei in different medial septum of the experimental groups. Each value represents the ±S.E.M. ##p<0.01 vs. normal group & ***p<0.001 vs. control group, respectively.

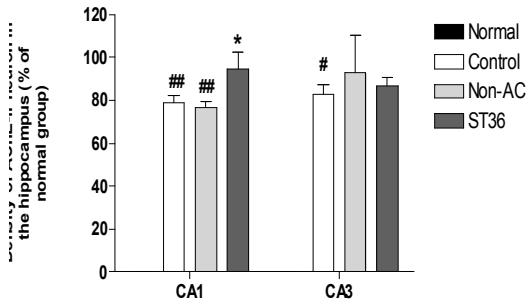

Fig. 3. The number of acetylcholine esterase (AChE) immunostained nuclei in different hippocampal CA1 and CA3 of the experimental groups. Each value represents the ±S.E.M. ##p<0.01 vs. normal group & *p<0.05 vs. control group, respectively.

Discussion

The present study demonstrated that TMT injections produced severe deficits in rat performance in a Morris water maze along with signs of neurodegeration, including decreased ChAT and AChE activity in the hippocampus and medial septum. Treatment of EA at acupoint ST36 attenuated TMT-induced learning and memory deficits in the maze and had a protective effect against TMT-induced decrease in ChAT and AChE positive neurons.

Intoxication with TMT leads to profound behavioral and cognitive deficits in both humans[27] and experimental animals[8,9]. In one reported case, postmortem examination revealed generalized chromatolysis of the neurons in the brain, spinal cord and spinal ganglia and neuronal necrosis in the Fascia Dentata and in the pyramidal cell layer of the hippocampus, cerebral cortex, basal ganglia and Purkinje cell layer of the cerebellum, findings similar to those described in experimental TMT intoxication[28]. Futhermore, behavioral studies have shown increased disruption in memory and learning deficits in TMT-intoxicated rats[29,30]. The Morris water maze is well-established paradigm for evaluating deficits in hippocampal-dependent memory and the MWM spatial learning task has been used in the validation of rodent models for neurocognitive disorders and for the evaluation of possible neurocognitive treatments[31]. The impairment in spatial learning produced by TMT in the current studies is consistent with previous reports of spatial learning impairments[13,14,32-34]. However, this study proved that spatial memory continued to improve in ST36 group during the training days compared to the control group. Also, the data of spatial probe trial demonstrated that electroacupuncture at ST36 protects against the TMT-induced decrease of the spatial retention, especially long-term memory.

The neuroprotective effects of electroacupuncture on the central acetylcholine system were also examined by histochemistry of hippocampal neurons. The degeneration of the cholinergic innervation from the basal forebrain to the hippocampal formation in the temporal lobe is thought to be one of the factors determining the progression of memory decay, both during normal aging and AD[19]. The best available marker for cholinergic neurons in the basal forebrain is ChAT activity. ChAT synthesizes the neurotransmitter acetylcholine, basal forebrain and the cortex, hippocampus, medial septum and amygdala. A significant reduction in ChAT activity in the postmortem brains of demented patients has been reported. In addition, there is a 20-50% decrease in ChAT activity in the hippocampus of the TMT-induced rats. However, electroacupuncture at ST36 modulates the function of these neurons and plays a role in their maintenance by preventing the TMT-induced decrease in ChAT activity[35,36]. The present results show that electroacupuncture exerts beneficial effects on cholinergic neurotransmission in the brain by increasing the hippocampus ChAT activities.

In summary, treatment of EA attenuated TMT-induced learning and memory deficits in the Morris water maze and had a protective effect against TMT-induced decreased in cholinergic neurons. EA in ST36 is thus a good candidate for further investigations that may ultimately result in its clinical use.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. R11-2005-014).

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